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Brahma is required for cell cycle arrest and late muscle gene expression during skeletal myogenesis

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Esther Schnapp

1st Editorial Decision 18 February 2015

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is copied below as well as referee cross-comments.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also pinpoint missing controls and suggest several additional experiments to strengthen the study, which I will not repeat here. Upon cross-commenting on each others' reports, all referees agree that the crucial experiments should be performed in primary myoblasts, and we agree that the proposed, specific mechanisms by which Brm1 controls myoblast differentiation should be confirmed in primary cells. All referees also note that the discrepancies with the recently published paper must be discussed.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the issue further.

In your case, I think it makes more sense to publish your manuscript as a normal article instead of a short report with a maximum of 5 figures. EMBO reports has decided recently to publish also longer articles from now on. There are no length limits on articles, but please change the reference style to the numbered EMBO reports style.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, along with the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS:

Referee #1:

In this paper, Albini et al. have analyzed the individual roles of the SWI/SNF complex ATPase subunits Brg1 and Brm in skeletal muscle differentiation and regeneration. They claim that while Brg1 is involved in the activation of early differentiation genes, Brm1 regulates both the cell cycle exit by inhibiting proliferation-associated genes, such as the key cell cycle regulator Cyclin D1, and activation of late muscle differentiation genes. In accordance with these data obtained in cellulo, Brm-null mice show impaired skeletal muscle regeneration concomitant to aberrant proliferation of muscle stem cells (satellite cells) and delayed myofiber formation. These data suggest that Brm1 participates in activating and repressive SWI/SNF complexes. Overall the experiments reported support the conclusion of the authors. This work is original and of interest to those in the fields of chromatin remodelling, proliferation, differentiation and regeneration. There are a number of concerns that the authors are invited to resolve before the manuscript could be accepted for publication. Thus, the following few points are intended as constructive suggestions to improve the manuscript.

Comments:

- Fig 1A: Brg1 and Cdk4 western blots seem overexposed. It would be better to show less exposed ones. Specify in the legend how many times the experiment was performed.

- Fig 1B: Is it possible to show higher magnification pictures?

- Fig 1C: Specify please time post-transfection. It would be better to add a diagram of the experimental setup, as in Figure 3A.

- What is the effect of Brm siRNA on Brg1 protein level and vice versa? Figure 2G suggests that siBrg1 lowers Brm protein level. Is this reproducible? Can you comment on this?

- Fig 1 and 2: As for the effect of siBrm on cell proliferation, the shown figure panels do not reflect this.

- Page 12: the authors stated this: "Immunofluorescence analysis showed that the large proportion of siBrm myoblasts that expressed Cyclin D1 continued to incorporate EdU, as a reflection of their failure to withdraw from the cell cycle (Fig. 2E-G).", and on page 13: "...indicating that Cyclin D1 repression is important for cell cycle exit and activation of the myogenic program at early stages of myoblast differentiation. ». However, in siBRM condition, cells in DM do express both Cyclin D1 and Myogenin (Fig 2G), suggesting a cell cycle exit of at least a subset of cells (those expressing Myogenin). In addition, commenting Fig 2A, the authors claimed: "It is formally possible that the latter population derives from cells in which Brm was not efficiently depleted ». Thus, is it possible to perform Brm-MyHC co-IF in siBrm vs control, to check this?

- Fig 2G: WB is overexposed! Show please less exposed ones if possible.

- Fig 3: what is the efficiency of siCyclin D1?

- The use of Actn3 allover the manuscript is not explained enough.

- Figure 4: Is the shRNAs efficiency monitored? If yes, please comment.

- Please discuss more the possible Brm-recruiting transcription factors, based on the literature and the transcriptomic data (Fig 2).

- Figure legends generally miss important information. They must be improved with more details.

- Specify the number of experiments in each figure legend please, when missing.

- Fig 1A and main text: « Brm protein levels were progressively upregulated during C2C12 differentiation »: specify that it is also transcriptional, based on Fig 1C, if significant.

- Fig 1F legend is missing.

- Fig 2G: Why Myogenin neither Actn3 WB results are not discussed in the main text?

- Fig S3 would be better shown as a main figure.

- Page 12: please comment more the choice of H3K27 tri-methylation mark here.

- Please specify if the siRNA and shRNA experiments were performed with more than one siRNA.

- Page 13 : change "siRNAi » into « siRNA ».

Referee #2:

1. Does this manuscript report a single key finding? One sentence to describe.
YES. Brm regulates myogenic differentiation at two distinct stages: repression of Cyclin D1 to allow cell cycle exit and subsequent activation of late differentiation genes.

2. Is the reported work of significance?
YES.

3. Is it of general interest to the molecular biology community? One sentence to describe why.
YES. The authors study the direct regulation of a critical cell cycle mediator and the downstream effects on gene expression, cell cycle proliferation and differentiation, and they also show differences between the highly-related ATPases Brm and Brg1.

4. Is the single major finding robustly documented using independent lines of experimental evidence?
YES.

Summary of review (major findings, overall impressions, strengths/weaknesses of manuscript)
The authors do an excellent job combining functional genomic approaches (gene expression profiling, bioinformatics) together with specific functional assays (EdU incorporation, myogenic differentiation, qRT-PCR) in order to explore the overlap and exclusive roles of Brm and Brg1 during myogenesis. The manuscript was easy to follow with clear goals, rationales for proposed experiments and valid interpretations of results. Although some work is shown using in vivo and ex vivo models, this manuscript would be of greater interest if more investigations were made in a relevant model (in vivo/ex vivo). Although C2C12 act as an excellent model to study myogenic differentiation, using them to study aspects of cell cycle progression and arrest will be problematic and perhaps irrelevant: C2C12 are immortal and are not a fair representation of how the cell cycle proceeds in primary myoblasts.

1. Major claims and how significant are they? 2. Are the claims novel and convincing? 3. Are the claims appropriately discussed in the context of earlier literature?

The first highlight of paper made by the authors is that Brm regulates muscle differentiation at two distinct stages of skeletal myogenesis. This claim is further subdivided with the next two highlights which go into more detail describing the two distinct stages of control. The authors sought to improve our understanding of the distinct roles of Brm and Brg1 during skeletal myogenesis. However, the great proportion of this story is focused on Brm. This claim is novel as it demonstrates the unique and exclusive role Brm exerts at these two stages and its mutual exclusivity from family member Brg1. However, this finding is limited insofar as the model utilized. In C2C12, the exclusivity of Brm and Brg1 at these stages of myogenic differentiation is convincing, however further work will be required in a more relevant model to confirm the distinct roles of Brm from Brg1.

The second highlight of this paper is that Brm is required to repress Ccnd1 to allow downstream myoblast cell cycle arrest at the onset of differentiation. Ccnd1 is a candidate discovered through functional genomic approaches and validated in vitro and in vivo. Consistently, the in vitro and in vivo models demonstrate an over-expression of Ccnd1 transcripts in response to siBrm. Furthermore, this over-expression and the subsequent proliferation phenotype are rescuable with siCyclinD1, at least in vitro. As the literature has previously reported a role for Ccnd1 towards myoblast proliferation, this paper nicely demonstrates the importance (and mechanism) of Ccnd1 regulation. The authors should seek to improve this story by demonstrating the ability to rescue this phenotype in primary cells, rather than only with immortal myoblasts. Furthermore, a portion of the data seeks to demonstrate the direct regulation of Ccnd1 by Brm using ChIP assays. The authors presume that YY1 might be the recruitment mechanism to bring Brm to the Ccnd1 locus. The link between YY1 and PRC2 recruitment is not so well established, and YY1 ChIP-seq in C2C12 cells has essentially ruled out a widespread connection between the two (c.f. Lu et al. EMBO J 2013, PMID 23942234). Furthermore, the ChIP-seq data from Lu et al. shows no sign of YY1 binding at the Ccnd1 locus, in myoblasts and myotubes. Consultation of the publicly available ENCODE-Caltech ChIP-Seq for E2F4 and Myod show no binding peaks in the regions detailed in FigS3, either. In light of this, and short of demonstrating a clear recruitment mechanism for Brm at the Ccnd1 locus, the authors should at least discuss further how Brm, is recruited to the Ccnd1 promoter, and how this putative mechanism would avoid recruiting Brg1.

The next highlight made by the authors is that Brm is required to activate late muscle differentiation. This highlight was supported nearly exclusively in vitro with some supporting ex vivo and is novel as it demonstrates temporal roles of Brm and Brg1 during myogenic differentiation. Loss of either Brm or Brg1 has been shown to result in decreased myogenic differentiation. However unlike Brg1, the timing of Brm knock-down does not change the differentiation phenotype: Brg1 lost during early differentiation exhibit no impaired differentiation. Furthermore, the addition of myogenin is capable to rescue Brg1 loss but has no effect on Brm. This role of Brg1 in the activation of early muscle gene expression confirms what the literature has previously reported and the authors address this, however the temporal role of Brm has been unreported. Some data from WT and Brm null mice demonstrate decreased expression of late muscle differentiation genes and a requirement for Brm towards myogenic conversion. However similar rescue assays and timing of Brm/Brg1 loss that were performed in vitro should be considered for ex vivo studies in order to make the story more compelling and interesting.

The final highlight made by the authors is that Brm null mice show impaired muscle regeneration. This is demonstrated using mice models during resting conditions or in response to injury with subsequent H&E staining or eMHC. Qualitative and quantitative results are obtained that convincingly show impaired muscle regeneration in Brm null mice. Furthermore, valid questions regarding the use of constitutive Brm null mice and the specificity of this data to satellite cells are appeased. The investigations also performed ex vivo experimentation: namely assays of myogenic differentiation, myogenic conversion, and relative gene expression. These data further develop confidence and understanding of the essential role of Brm during adult muscle regeneration.

4. Who will be interested and why? 5. Does the paper stand out from others in the field? 6. Are the experimental data of sufficient quality to justify the conclusions?

This manuscript will be of interest to researchers/labs striving to further understand the complex regulation of myogenesis during adult muscle regeneration. The data presented fits well into the current literature and further highlights the importance of temporal gene expression and its regulation towards the delicate balance between myogenic proliferation, quiescence and differentiation. Although the data is of excellent quality and arrives at a novel and interesting conclusion, the manuscript does not stand out based on some inherent limitations. A large portion of the manuscript focuses on C2C12 myoblasts as a model to study cell cycle arrest. More efforts should be put towards replicating the C2C12 data with primary myoblasts/satellite cells data which should be relatively straightforward as the authors display the availability within their current set of methods. However, regardless of these limitations, the authors do an good job of interpreting the data and arrive at valid conclusions within the boundaries of their models.

Specific review of figures and results

Figure 1C
Inconsistent: Results state on page 9 "...more than 80% reduction of Brm and Brg1 transcripts and protein levels after 48 hours of DM..." but figure looks closer to 50% reduction of Brg1.

Figure 2B/3D
Inconsistent: Results state on page 9 that it is "...consistently observed a higher number of myoblasts in siBrm-treated myoblasts at the time of induction, as compared to siBrg1 and control samples" Yet both Fig2B and 3D show that there is no significant changes in EdU incorporation when cells are maintained in GM. The authors should comment the observation that there are more cells without there being more EdU+ cells.

Figure 2D: The number of cell cycle genes deregulated by siBrm at DM18h is 7% of 58, or 4 genes. The authors could name them all without taking too much space.

Figure S3
To convincingly demonstrate specificity, it is customary in ChIP experiments to also show the binding at a negative control locus, not bound by the factor of interest (Brm/Brg1, here), in addition to performing the ChIP with an irrelevant antibody.

Fig. S4: It would be informative to know what are the expression levels of Ccnd1 in the myogenic conversion experiment with MEFs (WT and Brm-/-). We expect the levels to be higher in the Brm-/-, as seen with siBrm in C2C12 cells.

Fig. 5C: The authors should comment on whether the fiber caliber differences in 'no-injury' mice are only observed in the TA muscle or also in other muscle groups of varying fiber composition and anatomical location. For example, are the soleus and diaphragm affected as much as the TA?

General comments and questions about the manuscript

Did the authors at any time perform double Brm/Brg1 knock-down? It would be interesting to see the effect on Cyclin D1 expression in a double knock-down as their data shows Ccnd1 annotated to be up-regulated in both siBrm and siBrg1 after 48 hours DM. FigS2 shows Ccnd1 expression upregulated after siBrg1 in DM48 (is this significant?). Fig2C also shows a small increase in relative Ccnd1 expression after siBrg1 in DM48.

What is Ccnd1 protein expression after siCyclinD1? The authors consistently observe Ccnd1 transcript levels, but it would be relevant to show the protein levels as well.

Figure 3D shows that loss of Ccnd1 has essentially no effect on EdU incorporation in growing cells. This is surprising given Ccnd1's known role towards G1/S-transition and considering that Ccnd1 down-regulation is presented as a key even for cell cycle exit and differentiation, in myoblasts.

The authors should comment on the discrepancy between the drastic decrease in differentiation in C2C12 with siBrm, and the somewhat milder regeneration deficit in Brm-/- animals.

Considering that the levels of Brm increase during differentiation, it would make sense to assume

that Brm over-expression leads to lower Ccnd1 expression. Does this also result in pre-mature and/or enhanced differentiation?

To summarize, a revised manuscript would ideally address the comments above, and include the following additional data:
-Ccnd1 western blots in C2C12 and in the myogenic conversion assay
-Results of over-expression of Brm and Brg1 in growing myoblasts
-Results of combined knock-down of brm and Brg1 on Ccnd1 expression at 48h of differentiation
-Revision or comment on the lack of effect of siCcnd1 on EdU labelling.
-Inclusion of a negative control locus for the ChIP experiments in Fig. S3.

Minor comments

At page 6 of the introduction, the following sentence is probably too general and needs to be clarified with regards to the cell type in question: "Despite the high degree of homology between the two subunits and their partial overlapping role, they have different expression profiles: Brg1 is expressed constitutively, whereas Brm levels fluctuates with increased expression in G0-arrested cells and in cells induced to differentiate;"

On page 9, the description of Fig 1D refers to 1E

Page 13: "siRNAi" should be simply "siRNA"

On page 14, the header "Brm but not Brg1 is required for the completion of myogenin downstream, late stages of muscle differentiation" is not very clear. Perhaps the author mean "for the completion of myogenesis events posterior to myogenin induction"

Page 17: the header "Brm null mice exhibit delayed muscle regeneration and Brm null satellite display intrinsic deregulation of cell cycle and impaired differentiation." should refer to 'satellite cells'.

Figure 4C stains for Actn3 but Fig4E,4G show quantifications of MyHC myotubes instead of Actn3. Which differentiation marker was used?

Fig. 5 C/D: the color code legend is missing

Fig. 5G/H: No statistical significance is indicated on the histograms?

Figure 5G: The relative number of cells does not add up to 100%. ~90% Pax7+ cells in WT mice, ~70% Pax7+ cells in Brm-/-.

Page 18: for the statement "in muscle satellite cells - the cellular effector of muscle regeneration (Brack & Rando, 2012; Yin et al, 2013)", the authors should be referencing the original work, not reviews.

Referee #3:

In this paper Albini et al., describe the possible significance of Brahma (Brm), one of the catalytic subunits of SWI/SNF complex, in the control of cell cycle arrest and in the regulation of late myogenic genes during muscle differentiation. By using both in vitro and in vivo approaches, the authors conclude that Brm is required for cell cycle exit prior to the activation of the myogenic program, while Brg1, the mutually exclusive catalytic subunit of the SWI/SNF complex, is essential only for the activation of early muscle genes. Accordingly, BRG1 knock-down performed after 6hrs in differentiation medium does not interfere with myoblasts differentiation, while Brm knock down at the same time point prevents differentiation. While the mechanism by which Brm regulates late myogenic genes is not described, the authors identify Cyclin D1 as the mediator of the Brm-induced cell cycle arrest in early steps of myogenesis. Overall with this work Albini et al. further clarify the role of Brm and Brg1 in the control of the myogenic program, describing a novel mechanism by

which Brm controls cell cycle arrest in the early step of differentiation and proposing an additional role in the regulation of late myogenic genes.

Specific comments:

Point 1) Recently Joliot et al., (Plos One 2014) found that BAF47 and BRG1 are both downregulated during myoblast differentiation, while in the early step of myogenesis both bind the Cyclin D1 promoter, with BAF47 playing the major role in cell cycle exit. By using the same cell line (C2C12 cells) and the same time points, in this manuscript

1) " the same levels of expression of Brg1 protein were detected in proliferating myoblasts and during the whole differentiation process" (Fig. 1A, this manuscript compared to Fig. 1B Joliot et al, 2014).

2) "Brm, and not Brg1, bound the regulatory elements of Cyclin D1" (Fig.S3 this manuscript compared to Fig.3A Joliot et al, 2014)

The authors should take in consideration Joliot's work and try to reconcile these discrepancies.

Is BAF47 part of the Brama-containing SWI/SNF complex? The biochemical definition of the complex would contribute to solve the discrepancies between the two papers. Is it possible that Brg1 is dispensable in the late phase of myogenesis due to its replacement with Brm, which is constantly upregulated during C2C12 differentiation (Fig. 1A)?

Point 2) The Authors claim that Brm regulates the exit from the cell cycle: this should be confirmed by showing a proliferation curve and a cell cycle analysis of cells silenced for Brm and Brg1.

Point 3) I have some concerns on the GEP analysis:

a) the Authors performed GEP only in siRNAs-silenced cells during differentiation. However, both Brm and Brg1 are expressed in proliferating myoblasts. GEP should include also proliferating cells.

b) the statistical analysis applied to GEP data is not described. What is the FDR of this experiment? Furthermore, a fold change of 1.3 is not biologically significant especially at mRNA level. The authors should include only genes with a fold change of 2. The complete list of differentially regulated genes should be included in a supplementary table, where genes commonly or specifically modulated by both subunits are clearly indicated. Which is the fold change of Cyclin D1 in Brm compared to Brg1 silenced cells? This data can clarify the contribution of each subunit to the control of Cyclin D1 expression in the early phase of differentiation. Accordingly, in Fig.S2 the Authors should include Cyclin D1 and myogenin level of expression at the DM 18 time point.

c) Fig.S1, Fig. 2D and related sentence: "we observed upregulation of cell cycle related genes only in siBrm myoblasts". In Fig.2D the % of cell cycle genes in siBrg1 cells is 0%. However, in Fig.S1 the GO analysis showed that cell cycle genes are also enriched in the downregulated genes for both siBrm and siBgr1 cells. How do the Authors explain this discrepancy? The Authors should reconsider the interpretation of these data upon the inclusion of additional proliferating samples and with a more appropriate analysis.

Point 4) Fig.4A The proposed model, based on Brm silencing at time 0 in DM, suggests a role for Brm in cell cycle arrest at the onset of myogenesis. Which is the level of cyclinD1 in cells Brm-silenced after 6 hrs in DM?

Point 5) Fig.4B: The inability of myogenin to rescue the differentiation program in Brm knock down cells is to be expected, considering that in both figure 3B and 4C, siBrm cells express myogenin at a level comparable to sictrl cells (Fig. 3B) and are not able to complete the differentiation program. Indeed, a more stringent rescue experiment should rather include another myogenic master gene (i.e. MyoD, as used in the Brm-null MEFs). Is myogenin also up in Brm-null MEF transfected with MyoD?

- In the Discussion the authors should de-emphasize the relevance of their in vivo findings. Brm null mice have a very weak muscle phenotype. The defective regeneration process upon CTX treatment resulted in a minimal delay in muscle regeneration. These data clearly indicate that there are compensatory mechanisms capable of regulating cell cycle arrest and late skeletal myogenesis in absence of Brm1. The scheme proposed in Fig.6. should be moved to the Supplementary Figures section: there is no evidence, in this manuscript, of the Brg1 and Brm binding ability on myogenin and actn3 regulatory regions.

Minor point: Throughout the manuscript there are numerous typos: i.e. in Fig.1A cycin D1 instead of Cyclin D1, heterogenic instead of heterogenous... The terminology 'genetic ablation' to describe RNA interference experiments is inappropriate.

1st Revision - authors' response 01 May 2015

Referee #1

We are glad to learn that this reviewer found that the experiments reported support the conclusions of the authors, and that our work is original and of interest to those in the fields of chromatin remodeling, proliferation, differentiation and regeneration.

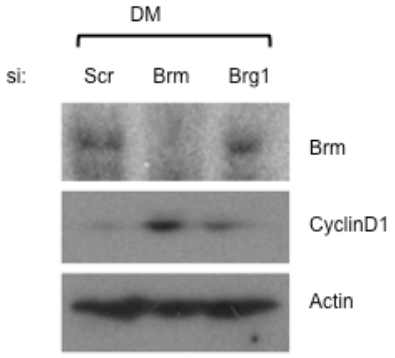
Comments:

- **Fig 1A: Brg1 and Cdk4 western blots seem overexposed. It would be better to show less exposed ones. Specify in the legend how many times the experiment was performed**.
  - RE: The experiment was performed 3 times, with the image shown in the figure 1A being representative of all western blots. Since Brg1 and cdk4 protein levels have been shown not to significantly change during the course of muscle differentiation, we believe that the WB in Fig. 1A fairly represents the pattern of protein levels in C2C12 myoblasts and myotubes.

- **Fig 1B: Is it possible to show higher magnification pictures?**
  RE: We can certainly show higher magnification pictures, although I have to point that pictures shown in Fig. 1 are already at high magnification, considering the necessity to show multiple nuclei typical of myotubes. If the reviewer specifies what particular detail should be shown at higher magnification, we would be happy to satisfy his/her request.

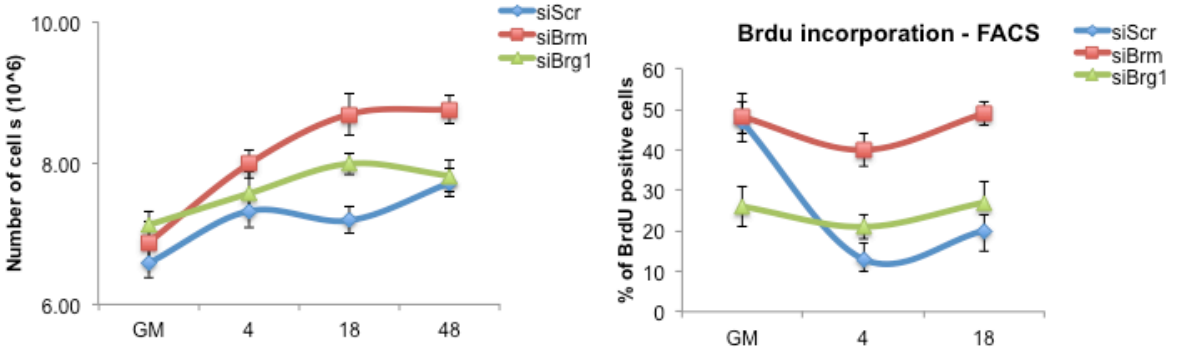
- **Fig 1C: Specify please time post-transfection. It would be better to add a diagram of the experimental setup, as in Figure 3A.**
  - RE: Time post-transfection is 48h, and diagram is included now in Fig. 1 of the new manuscript, as requested by the reviewer.

What is the effect of Brm siRNA on Brg1 protein level and vice versa? Figure 2G suggests that siBrg1 lowers Brm protein level. Is this reproducible? Can you comment on this?
RE: We have performed another WB using the same extracts saved from the original experiment shown in Fig. 2G. Pertinent to the specific reviewer question - *Figure 2G suggests that siBrg1 lowers Brm protein level* – we have measured the protein levels of Brm and two controls (cyclinD1 and tubulin). The WB is displayed below and shows no changes in Brm protein levels upon siBrg1. We have selected the Brm lane that has been now included in Fig. 3C of the revised Ms, in place of the original one.



- **Fig 1 and 2: As for the effect of siBrm on cell proliferation, the shown figure panels do not reflect this**.

  RE: The reviewer presumably refers to the picture in GM, and we agree that in this condition it is impossible to appreciate any difference in the proliferation rate between our experimental points, because of the presence of high mitogens (20% serum), which invariably promotes proliferation. Likewise, when cells reach confluence, prior to incubating them into mitogen-free differentiation medium (DM), confluence does not allow to appreciate "visible" difference in cell number between by phase contrast in siBrm, siBrg1 and siScr cells. However, when cells are incubated in DM we could notice an increase in cell density in siBrm cells that reflects a failure to exit the cell cycle. We have now included in Fig. 1E a new picture that is more representative of this phenomenon. To accurately monitor the proliferation rate of cells during GM and at different time points after incubation in DM, we have measured the proliferation curve by a) manual cell counting and b) FACS-assisted BrDU incorporation. The new data are shown below and reported in Fig. 2B of the revised Ms. They show an increased number of proliferating cells only in siBrm, following DM exposure. We have also clarified this point in the text of the revised Ms.



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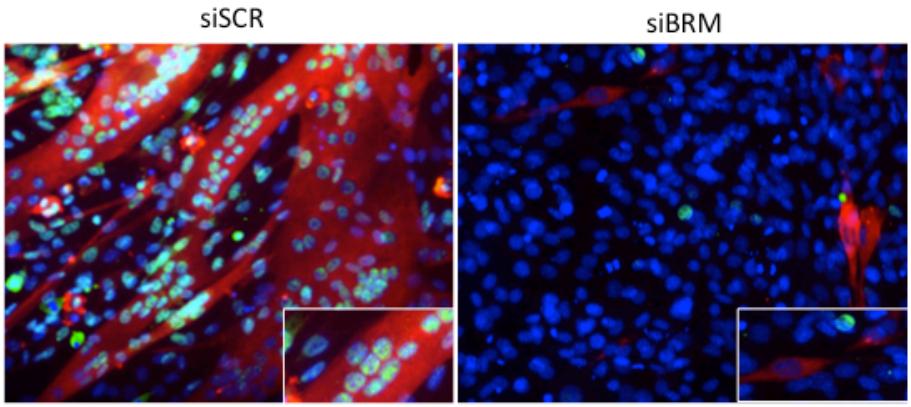
- **Page 12: the authors stated this: "Immunofluorescence analysis showed that the large proportion of siBrm myoblasts that expressed Cyclin D1 continued to incorporate EdU, as a reflection of their failure to withdraw from the cell cycle (Fig. 2E-G)." and on page 13: "...indicating that Cyclin D1 repression is important for cell cycle exit and activation of the myogenic program at early stages of myoblast differentiation. ». However, in siBRM condition, cells in DM do express both Cyclin D1 and Myogenin (Fig 2G), suggesting a cell cycle exit of at least a subset of cells (those expressing Myogenin). In addition, commenting Fig 2A, the authors claimed: "It is formally possible that the latter population derives from cells in which Brm was not efficiently depleted ». Thus, is it possible to perform Brm-MyHC co-IF in siBrm vs control, to check this?**

  RE: We followed the reviewer recommendation and performed IF for simultaneous detection of Brm/MyHC by co-staining in cells under our experimental conditions. Data show that sporadic MyHC-positive cells in siBrm samples do not express Brm, indicating that a small subset of cells might have undergone cell cycle exit, either because they were already committed to differentiation before siBrm or because of an alternative/compensatory mechanism operating in a fraction of myoblasts. These data are shown below to the reviewers, we do not feel to include them in the manuscript figures, as they would not add any substantial new information.



-**Fig 2G: WB is overexposed! Show please less exposed ones if possible**.

○ RE: As we commented above, also in this WB the experiment was performed 3 times, with the image shown in the figure 3C being representative of all WB. Indeed, in all exposures the difference in protein levels is clear, as compared to control loading Actin. However, we have replaced Brm WB with a new one, as indicated above (point #4).

- **Fig 3: What is the efficiency of siCyclin D1?**

RE: In Fig 4C of the revised Ms, qPCR shows the efficiency of Cyclin D1 transcript level reduction in GM and DM. In figure S3 we show now by immunofluorescence that the CyclinD1 protein levels are uniformely downregulated in all cultures.

The use of Actn3 all over the manuscript is not explained enough…

RE: Actn3 is a late muscle differentiation marker that was shown by microarray as highly downregulated following siBrm or siBrg1 at 48h DM and was therefore selected as an optimal "reference gene" for monitoring the effect of siBrm on muscle gene expression. This has been now clearly stated in the text.

- **Figure 4: Is the shRNAs efficiency monitored? If yes, please comment**.

RE: In all our experiments, we have routinely assessed the efficiency of siRNA by RTqPCR, and WB or IF, as shown in the figures.

- **Please discuss more the possible Brm-recruiting transcription factors, based on the literature and the transcriptomic data (Fig 2).**

RE: We have included this request in the discussion, by discussing the potential interactions with two previously identified partners of Brm – the Retinoblastoma gene product and Polycomb group.

- **Figure legends generally miss important information. They must be improved with more details**.

RE: We apologize for having omitted important information for this reviewer, and have now added important details and information in the figure legend.

- **Specify the number of experiments in each figure legend please, when missing**.

RE: We have added the number of experiments, when missing.

- Fig 1A and main text: « Brm protein levels were progressively upregulated during C2C12 differentiation »: specify that it is also transcriptional, based on Fig 1C, if significant.

RE: We changed that in the text.

Fig 1F legend is missing.

RE: We added legend 1D that was missing and fixed the legend 1F.

-**Fig 2G: Why Myogenin neither Actn3 WB results are not discussed in the main text?**

RE: Myogenin is an early muscle differentiation marker that was selected as an optimal "reference gene" for monitoring the effect of siBrm or siBrg1 on early myogenesis. As mentioned above, Actn3 is a late muscle differentiation marker that was shown by microarray as highly

downregulated following siBrm or siBrg1 at 48h DM. While this has been now clearly stated in the text during the description of the qRT-PCR validation of the microarray data, in the case of this WB the protein levels of myogenin and Actn3 are simply used as control/validation of the effects of siBrg1 or siBrm. Indeed, this figure (now Fig. 3C of the revised Ms) is aimed at assessing the different impact of siBrm vs siBrg1 on CyclinD1 protein levels.

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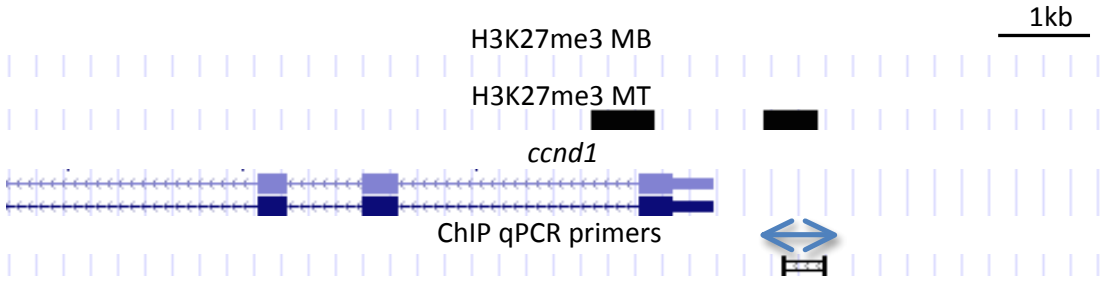
Fig S3 would be better shown as a main figure.

- RE: We thank the reviewer for this suggestion that we have welcomed. ChIP for CyclinD1 has been introduced now in Fig. 3 of the revised Ms (panel D)

- Page 12: **please comment more the choice of H3K27 tri-methylation mark here**.

RE: Based on publicly available ENCODE data, there is enrichment of H3K27me3 at ccnd1 regulatory regions in myotubes but not in myoblasts. H3K27me3 is a marker of inhibition of gene expression; therefore this is concordant with *ccnd1* repression in myotubes – see screenshot below. Also H3K27me3 reflects the functional interaction between SWI/SNF and Polycomb complexes suggested in previous works to promote repressive chromatin at target genes (Ho et al. 2011)



Please specify if the siRNA and shRNA experiments were performed with more than one siRNA.

- RE: siRNAs are a pool of 4 siRNAs (smartpool). shRNA were not used on our experiments.

Page 13 : change "siRNAi » into « siRNA »

RE: OK

Referee #2

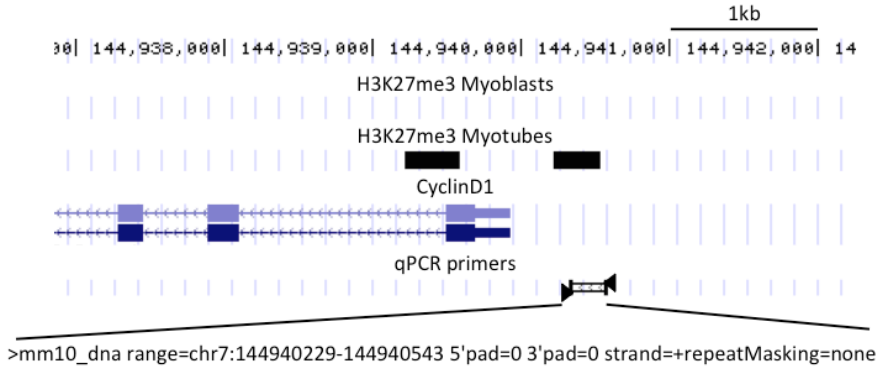
We appreciate that this reviewer found that “the authors did an excellent job combining functional genomic approaches (gene expression profiling, bioinformatics) together with specific functional assays (EdU incorporation, myogenic differentiation, qRT-PCR) in order to explore the overlap and exclusive roles of Brm and Brg1 during myogenesis” and that “the manuscript was easy to follow with clear goals, rationales for proposed experiments and valid interpretations of results”.

Comments

The authors should seek to improve this story by demonstrating the ability to rescue this phenotype in primary cells, rather than only with immortal myoblasts. Furthermore, a portion of the data seeks to demonstrate the direct regulation of Ccnd1 by Brm using ChIP assays. The authors presume that YY1 might be the recruitment mechanism to bring Brm to the Ccnd1 locus. The link between YY1 and PRC2 recruitment is not so well established, and YY1 ChIP-seq in C2C12 cells has essentially ruled out a widespread connection between the two (c.f. Lu et al. EMBO J 2013, PMID 23942234). Furthermore, the ChIP-seq data from Lu et al. shows no sign of YY1 binding at the Ccnd1 locus, in myoblasts and myotubes. Consultation of the publicly available ENCODE-Caltech ChIP-Seq for E2F4 and Myod show no binding peaks in the regions detailed in FigS3, either. In light of this, and short of demonstrating a clear recruitment mechanism for Brm at the Ccnd1 locus, the authors should at least discuss further how Brm is recruited to the Ccnd1 promoter, and how this putative mechanism would avoid recruiting Brg1.

RE: We agree with the reviewer on replicating our data in primary muscle cells, and we have now included data showing retained expression of ccnd1 in BRM-/- satellite cells upon differentiation

stimuli.
For what concerns ccnd1 regulation, based on publicly available datasets, there is H3K27me3 enrichment in *ccnd1* regulatory regions in myotubes, but not in myoblasts, as shown in the picture below and by our ChIP-qPCR analysis in Fig. 3D. This suggests an involvement of PRC2 in repressing ccnd1 expression. Indeed, ChIP-PCR by Blais et al., 2007 showed EZH2 presence at ccnd1 promoter in myotubes and not myoblasts. Finally, bioinformatics search revealed the presence of E2F4-6 and YY1 binding sites in the region of *ccdn1* promoter that was amplified by the primers used in our ChIP analysis – see figure below. This evidence suggests that Brm can be recruited through interactions with two previously proposed partners – Rb, via repressive E2F binding sites, and/or Polycomb, via YY1 binding sites. We have included this discussion in the text of the revised Ms.



>mm10_dna range=chr7:144940229-144940543 5'pad=0 3'pad=0 strand=+repeatMasking=none

GGGGTGCAAGGACACCCTGTTCTTAAACCGGGAGAATGGGTGCGTTTCCGAGTACGCCACGAGGCACCC
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CAGGAACTGTGTAACACCCGAAGATGCCAGACGAGCCCTAAGCTCTCGTGCAGGCTCCATCCTGGCGCA
CTGGGGTGGTTGCAAAGGGCCAGAGGGGTATCTGAACTTTAAAGGATCCCCAGCCTAGCATGCGCTCGC
TCAAAAATAAAATAAAACCCCGAAAATTCCAGCAACA

AACCGGGAGA	E2F4 Binding Site	1 strand
AACCGGGAGAA	E2F6 Binding Site	1 strand
AGGATGGAGCC	E2F6 Binding Site	-1 strand
CCCATT	YY1 Binding Sites	-1 strand and 1 strand
GGCTCCATCCTG	YY1 Binding Site	-1 and 1 strand

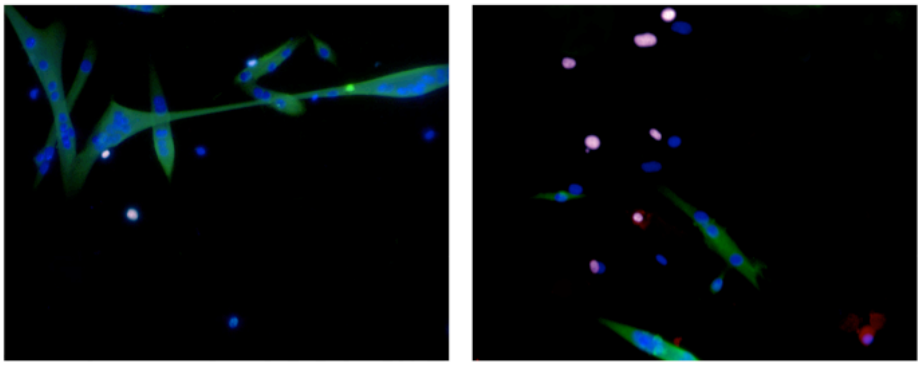
The next highlight made by the authors is that Brm is required to activate late muscle differentiation….. This highlight was supported nearly exclusively in vitro with some supporting ex vivo and is novel as it demonstrates temporal roles of Brm and Brg1 during myogenic differentiation…….some data from WT and Brm null mice demonstrate decreased expression of late muscle differentiation genes and a requirement for Brm towards myogenic conversion. However similar rescue assays and timing of Brm/Brg1 loss that were performed in vitro should be considered for ex vivo studies in order to make the story more compelling and interesting.

RE: We have decided to work with C2C12 for multiple reasons:

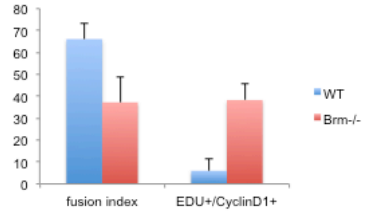
1) They are a well-established in vitro model for muscle differentiation;
2) Their differentiation is more synchronous and uniform, as compared to primary myoblasts, and provide a better setting for gene expression analysis, as proven by Asp et al 2011;
3) They have been widely used in the past so we could look at ENCODE genome-wide sequencing data, such as H3K27me3 ChIPSeq, to better understand the mechanism;
4) The gene expression profile between C2C12 and primary myoblasts highly correlates (Blais et al., 2005);
5) There is strong concordance between MYOD binding sites between C2C12 and primary muscle cells (Cao et al., 2010);
6) They resist to manipulation (like siRNA and transfection, while satellite cells and primary myoblasts do not survive to even a single siRNA).

As requested by this reviewer and reviewer 3 we attempted to repeat all the siRNA experiments in primary myoblasts as well as in primary satellite cells; however, we noticed that transfection largely impaired the survival of either type of primary cells, with the residual surviving cells not being able to reach a significant amount of cells to analyze, as we did with C2C12. Therefore, we could not draw

significant conclusion from these experiments. However, we could replicate in primary satellite cells from Brm null mice all the major findings reported in the original version of our manuscript.
The new data on satellite cells from WT vs Brm null mice at “low density” are displayed below and are now reported in Suppl. Fig. 5 and show that upon differentiation stimuli, Brm -/- satellite cells have an impaired muscle differentiation, continue to express Cyclin D1 and retain the proliferation ability, as shown by the presence of EdU+ and CyclinD1+ cells after 48hrs of differentiation stimulus.



MyHC/D1/EDU/Dapi



A large portion of the manuscript focuses on C2C12 myoblasts as a model to study cell cycle arrest. More efforts should be put towards replicating the C2C12 data with primary myoblasts/satellite cells data which should be relatively straightforward as the authors display the availability within their current set of methods. However, regardless of these limitations, the authors do a good job of interpreting the data and arrive at valid conclusion within the boundaries of their models.

RE: We agree with the reviewer and made as many experiments as possible considering the fragile nature of satellite cells and primary myoblasts. We showed that satellite cells isolated from Brm KO mice have elevated levels of cyclin D1 (*ccnd1*), show impaired differentiation ability and cell cycle exit. We note that the same conclusion was derived from analysis of Brm KO satellite cells *in vivo*, as shown in Fig. 6, and are corroborated by experiments of myogenic conversion of BRM null MEFs (Fig. S4). Moreover, we show that reintroduction of Brm in satellite cells isolated from Brm KO mice restore their ability to form multinucleated myotubes (Fig. S4D). We believe that our data on two parallel models of myogenesis (primary satellite cells in vivo and ex vivo, and fibroblast conversion into muscle) provide robust evidence in support of our model derived from C2C12 cells.

Specific review of figures and results

Figure 1C. Inconsistent: Results state on page 9 "...more than 80% reduction of Brm and Brg1 transcripts and protein levels after 48 hours of DM..." but figure looks closer to 50% reduction of Brg1.

RE: the reviewer is correct, as this was a mistake made during the data assembly. We have replaced the graphs with a more representative experiment in fig. 1E.

Figure 2B/3D. Inconsistent: Results state on page 9 that it is "...consistently observed a higher number of myoblasts in siBrm-treated myoblasts at the time of induction, as compared to siBrg1 and control samples"

RE: We improperly used “time of induction” to emphasize that we this phenomenon upon differentiation, therefore we have rephrased the concept in the text.

Yet both Fig2B and 3D show that there is no significant changes in EdU incorporation when cells are maintained in GM. The authors should comment the observation that there are more cells without there being more EdU+ cells.

RE: We clarified that the effect of Brm on cell cycle is only observed upon DM condition, not in GM where there is no significant increase in EDU+ cells (Fig. 2A/B)

Figure 2D: The number of cell cycle genes deregulated by siBrm at DM18h is 7% of 58, or 4 genes. The authors could name them all without taking too much space.

RE: We named them - Fgf, Vegfc, ccng1, ccnd1 - and we included them in the text.

Figure S3. To convincingly demonstrate specificity, it is customary in ChIP experiments to also show the binding at a negative control locus, not bound by the factor of interest (Brm/Brg1, here), in addition to performing the ChIP with an irrelevant antibody.

RE: We argue that in this case, comparison of ChIP data in GM and DM conditions provide an internal control by showing specific enrichment of Brm on chromatin of cyclin D1 promoter in GM vs DM.

Fig. S4: It would be informative to know what are the expression levels of Ccnd1 in the myogenic conversion experiment with MEFs (WT and Brm-/-). We expect the levels to be higher in the Brm-/-, as seen with siBrm in C2C12 cells.

RE: As the reviewer predicted, *ccnd1* expression is higher in Brm-/- MEF-derived myotubes compared to BRM+/+ MEF-derived myotubes – see image below only for reviewers.

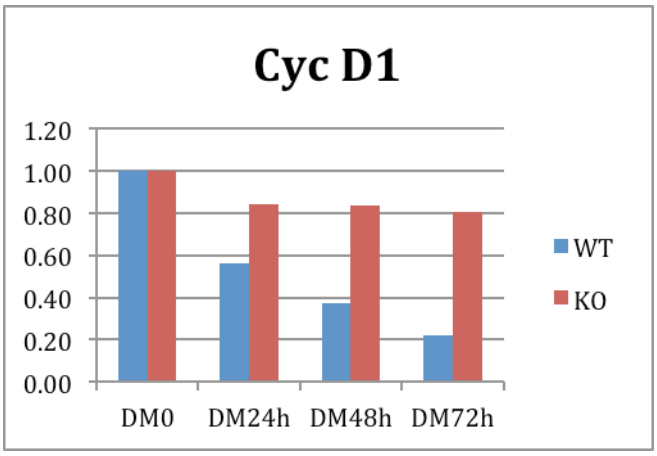


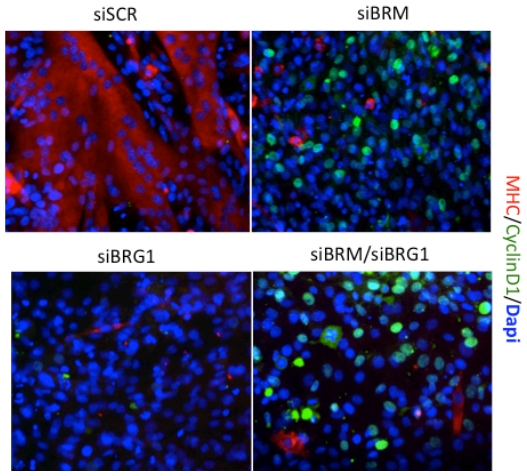
Fig. 5C: The authors should comment on whether the fiber caliber differences in 'no-injury' mice are only observed in the TA muscle or also in other muscle groups of varying fiber composition and anatomical location. For example, are the soleus and diaphragm affected as much as the TA?

RE: We have also observed the same differences in gastrocnemius muscles, and this is now indicated as data now shown. However, we are willing to include them in the Ms, if requested by the reviewer.

General comments and questions about the manuscript

Did the authors at any time perform double Brm/Brg1 knock-down? It would be interesting to see the effect on Cyclin D1 expression in a double knock-down as their data shows Ccnd1 annotated to be up-regulated in both siBrm and siBrg1 after 48 hours DM. FigS2 shows Ccnd1 expression upregulated after siBrg1 in DM48 (is this significant?). Fig2C also shows a small increase in relative Ccnd1 expression after siBrg1 in DM48.

RE: We performed double knock-down shows impaired differentiation and presence of CyclinD1+ cells as expected, with no significant differences as compared to siBrm. The data are shown below for the reviewers, but we are happy to include them in the Ms, if requested by the reviewer.



What is Ccnd1 protein expression after siCyclinD1? The authors consistently observe Ccnd1 transcript levels, but it would be relevant to show the protein levels as well. The protein expression of CyclinD1 is greatly reduced upon siCyclinD1 as shown by the absence of signal in IF.

RE: We have included a figure showing the nuclear protein level of CyclinD1 by IF in the new version of the Ms. We believe that in this case IF is more accurate than WB, as it allows to determine how uniform was the efficiency of siRNA in the cell population. This figure has been included in the revised Ms as Fig.S3, and it is shown below.

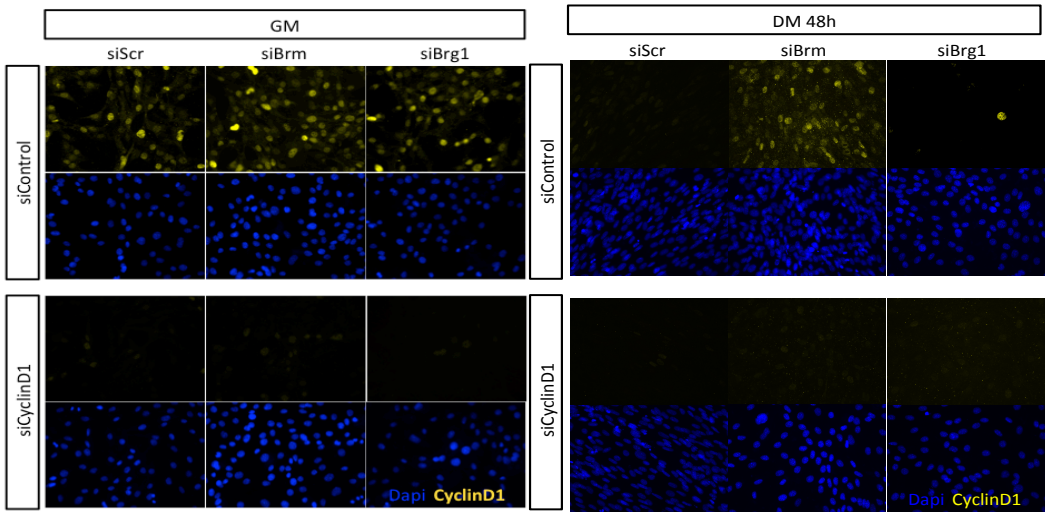


Figure 3D shows that loss of Ccnd1 has essentially no effect on EdU incorporation in growing cells. This is surprising given Ccnd1's known role towards G1/S-transition and considering that Ccnd1 down-regulation is presented as a key even for cell cycle exit and differentiation, in myoblasts. See answer above… We clarified that the effect of Brm on cell cycle is only observed upon DM condition, He does not refer to Brm role, but to the siCyclinD1 effect itself.

RE: We agree with the reviewer that lack of effect on EdU incorporation in GM after CyclinD1 silencing is apparently surprisingly. However, the explanation of this data resides in the timing of EdU pulse/incorporation, relative to the timing of siRNA. Indeed, siCyclinD1 was invariably performed in C2C12 myoblasts in GM, and the 6-hour EdU pulse was done in GM only 18 hours after the siRNA – a time that is likely incompatible with a protein downregulation that could affect cell cycle. By contrast, in C2C12 cells cultured in DM, there was enough time to downregulate CyclinD1 (indeed Fig. 4C shows that transcripts levels in DM are than in GM) to see the effect of siCyclinD1 on EdU incorporation. As the scope of this experiment was to evaluate the impact of siCyclinD1 on EdU incorporation of siBrm C2C12 cells in DM, we believe that the data fit with our conclusions.

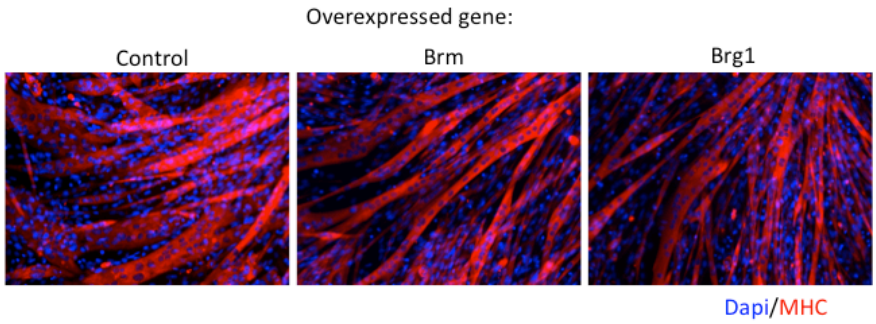
The authors should comment on the discrepancy between the drastic decrease in

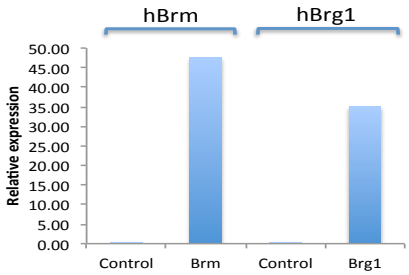
differentiation in C2C12 with siBrm, and the somewhat milder regeneration deficit in Brm-/- animals.

RE: It is possible that compensatory mechanisms in vivo account for the milder phenotype, although we note again that in vivo data support the importance of Brm in regulating cell cycle and differentiation of satellite cells. Probably, a more severe phenotype can be revealed by repeated injuries, and we are currently setting this type of analysis, which however will be a matter of an independent study.

Considering that the levels of Brm increase during differentiation, it would make sense to assume that Brm overexpression leads to lower Ccnd1 expression. Does this also result in pre-mature and/or enhanced differentiation?

RE: Overexpression experiments have been performed but did not shown any significant difference as compared to control, as shown in the figure below – only for reviewer.





Minor comments

At page 6 of the introduction, the following sentence is probably too general and needs to be clarified with regards to the cell type in question: "Despite the high degree of homology between the two subunits and their partial overlapping role, they have different expression profiles: Brg1 is expressed constitutively, whereas Brm levels fluctuates with increased expression in G0-arrested cells and in cells induced to differentiate;"

RE: We have re-phrased this sentence, and specified that it refers to specific cell types described in the references.

On page 9, the description of Fig 1D refers to 1E

RE: Fixed

Page 13: "siRNAi" should be simply "siRNA"

RE: Fixed

On page 14, the header "Brm but not Brg1 is required for the completion of myogenin downstream, late stages of muscle differentiation" is not very clear. Perhaps the author mean "for the completion of myogenesis events posterior to myogenin induction".

RE: We changed the header with “Brm but not Brg1 is required for the completion of muscle differentiation” since completion already refers to posterior genes to myogenin induction.

Page 17: the header "Brm null mice exhibit delayed muscle regeneration and Brm null satellite
display intrinsic deregulation of cell cycle and impaired differentiation." should refer to 'satellite cells'.

RE: Fixed

Figure 4C stains for Actn3 but Fig. 4E, 4G show quantifications of MyHC myotubes instead of Actn3. Which differentiation marker was used?
RE: We changed the graphs legend. Since Actn3 is a reliable marker to check for terminal differentiation, we used it the same way we use MyHC and we calculated the fusion index by counting the total number of nuclei included in actn3+ myotubes versus the total number of nuclei.

Fig. 5 C/D: the color code legend is missing
RE: Fixed

Fig. 5G/H: No statistical significance is indicated on the histograms?
RE: Fixed

Figure 5G: The relative number of cells does not add up to 100%. ~90% Pax7+ cells in WT mice, ~70% Pax7+ cells in Brm
RE: Fixed

Page 18: for the statement "in muscle satellite cells - the cellular effector of muscle regeneration (Brack & Rando, 2012; Yin et al, 2013)", the authors should be referencing the original work, not reviews.
RE: It is difficult to point to one single manuscript that demonstrated that satellite cells are the cellular effectors of muscle regeneration, since this conclusion has been established by combining information of several papers. This is why we decided to cite reviews. However, upon reviewer request we have included the reference of the initial description of satellite cells by Mauro, 1961.

Referee #3

This reviewer found that " overall with this work Albini et al. further clarify the role of Brm and Brg1 in the control of the myogenic program, describing a novel mechanism by which Brm controls cell cycle arrest in the early step of differentiation and proposing an additional role in the regulation of late myogenic genes"

Comments:

Point 1) Recently Joliot et al., (Plos One 2014) found that BAF47 and BRG1 are both downregulated during myoblast differentiation, while in the early step of myogenesis both bind the Cyclin D1 promoter, with BAF47 playing the major role in cell cycle exit. By using the same cell line (C2C12 cells) and the same time points, in this manuscript: 1) " the same levels of expression of Brg1 protein were detected in proliferating myoblasts and during the whole differentiation process" (Fig. 1A, this manuscript compared to Fig. 1B Joliot et al, 2014). 2) "Brm, and not Brg1, bound the regulatory elements of Cyclin D1" (Fig.S3 this manuscript compared to Fig.3A Joliot et al, 2014). The authors should take in consideration Joliot's work and try to reconcile these discrepancies.
RE: First of all, we thank the reviewer for reminding us about this important paper, which indeed we were aware of, but we inadvertedly omitted to cite and discuss. We have now included this citation and discussed potential implications. Regarding the difference in the expression levels of Brg1, this could be due to the time points analyzed, as Joliot et al. monitored the expression of Brg1 until 72h of differentiation, whereas we monitored it until 48hrs. However we monitored Brg1 relative gene expression (data not shown) and we observed downnregulation of Brg1 mRNA levels at the end of differentiation suggesting that Brg1 could decrease at the late stages, when it is not longer required for myogenesis.

As for the Brg1 recruitment on cyclin D1 promoter reported by Joliot et al., we indeed observed a minor Brg1 enrichment, as compared to Brm. If we do the same ratio Brg1/IgG in our DM condition (0.021/0.003), it seems that Brg1 is recruited 7 fold more over IgG, however the recruitment is extremely poor when compared to Brm recruitment (almost 100 fold more Brm than Brg1). As Joliot

et al. did not perform a ChIP with Brm antibodies the data are not comparable and would be therefore difficult to discuss.

Is BAF47 part of the Brama-containing SWI/SNF complex? The biochemical definition of the complex would contribute to solve the discrepancies between the two papers. Is it possible that Brg1 is dispensable in the late phase of myogenesis due to its replacement with Brm, which is constantly upregulated during C2C12 differentiation (Fig. 1A)?

RE: We don't think that data on SWI/SNF composition are matter of discrepancy, because SWI/SNF complexes undergo dynamic changes in composition, and Brg1- and Brm-based SWI/SNF complexes can be simultaneously present in the same cell. Because BAF47 is an "invariable and obligatory" SWI/SNF component, it is likely that BAF47 can be associated with distinct Brg1- and Brm-based complexes. However, in their Ms Joliot et. al., did not address the presence of Brm in the BAF47 complex, making problematic a further discussion of this issue.

Point 2) The Authors claim that Brm regulates the exit from the cell cycle: this should be confirmed by showing a proliferation curve and a cell cycle analysis of cells silenced for Brm and Brg1

RE: We have performed a proliferation curve and a BrdU-FACS analysis to substantiate the role of Brm in regulating cell cycle exit. Data are now shown in Fig. 2 (also shown in reviewer 1 response).

Point 3) I have some concerns on the GEP analysis:
a) the Authors performed GEP only in siRNAs-silenced cells during differentiation. However, both Brm and Brg1 are expressed in proliferating myoblasts. GEP should include also proliferating cells.

RE: While we agree with the reviewer that GEO in myoblasts is also interesting, we note that this paper focuses entirely on the role of BRM and BRG1 during myogenic differentiation and not in proliferating cells. To be consistent with this, in the paper we show the results of the GEO analysis only in differentiating muscle cells. The GEO analysis in proliferating myoblasts does not show any impairment in muscle differentiation since the differentiation program has not started yet (data not shown).

b) the statistical analysis applied to GEP data is not described. What is the FDR of this experiment? Furthermore, a fold change of 1.3 is not biologically significant especially at mRNA level. The authors should include only genes with a fold change of 2.

RE: We acknowledge that 1.3 is not a high fold change; however, silencing by RNA interference does not eliminate completely the protein levels, but just reduces them. Therefore, we did not predict a drastic change in gene expression. Furthermore, microarray is not very sensitive flattening the fold change of gene expression. For this reason, we performed qPCR, which is more sensitive, to validate the differential expression in siBRM and siBRG1 and we confirmed that the fold change is higher in qPCR analysis than in the microarray, as expected. More importantly, the biological relevance of the differential expression is not given by the number of the fold change, but by the phenotypic and functional analysis. The impaired muscle differentiation shown by in siBRM and siBRG1 is a very clear indication that the gene expression alteration in siBRM and siBRG1 is crucial for the muscle differentiation program, even though the fold change by microarray is not that stringent. Finally, microarray analysis identified a Brm target gene – CyclinD1 – whose regulation by Brm and functional relevance in the control of the cell cycle are demonstrated throughout the all paper, further indicating that accuracy of microarray data.

The complete list of differentially regulated genes should be included in a supplementary table, where genes commonly or specifically modulated by both subunits are clearly indicated. Which is the fold change of Cyclin D1 in Brm compared to Brg1 silenced cells? This data can clarify the contribution of each subunit to the control of Cyclin D1 expression in the early phase of differentiation. Accordingly, in Fig.S2 the Authors should include Cyclin D1 and myogenin level of expression at the DM 18 time point.

RE: We agree with the reviewer on this important point, and have included a qRT-PCR comparison of fold change of several differentially regulated genes, at the indicated time points, in Fig.S2 of the revised Ms. As for the complete list of genes, it can be easily retrieved from GEO data we uploaded. However, we added a table with the list of these genes, as requested by the reviewer, as well as a graph with the level of expression of cyclinD1 and myogenin at DM18h.

c) Fig.S1, Fig. 2D and related sentence: "we observed upregulation of cell cycle related genes

only in siBrm myoblasts". In Fig.2D the % of cell cycle genes in siBrg1 cells is 0%. However, in Fig.S1 the GO analysis showed that cell cycle genes are also enriched in the downregulated genes for both siBrm and siBgr1 cells. How do the Authors explain this discrepancy? The Authors should reconsider the interpretation of these data upon the inclusion of additional proliferating samples and with a more appropriate analysis.

RE: The sentence mentioned by the reviewer **"we observed upregulation of cell cycle related genes only in siBrm myoblasts"** refers to upregulated genes at the 18 hrs time point, where indeed we observe only upregulated cell cycle genes in siBrm samples. However, as the reviewer noticed, at 48 hrs also siBrg1 samples show upregulated cell cycle genes. As the reviewer pointed out, downregulated genes were found in siBrg1 at 18hrs time point; however, we did not further analyzed these genes, because we focused on cell cycle genes (and in particular CyclinD1), given the specific phenotype of siBrm myoblasts, which fail to arrest the cell cycle in DM. We apologize for not clarifying this in the original verison of the Ms. This has been made clear in the text.

Point 4) Fig.4A The proposed model, based on Brm silencing at time 0 in DM, suggests a role for Brm in cell cycle arrest at the onset of myogenesis. Which is the level of cyclinD1 in cells Brm-silenced after 6 hrs in DM?

RE: 6hrs after the addition of differentiation media the difference of cyclinD1 levels are unlikely as dramatic as at 48hrs, since not all cells exit the cell cycle synchronously once incubated in differentiation media (DM). Indeed, we performed a time course by WB, showing progressive decline of cyclinD1 level during myogenic differentiation, and complete absence of the proteins only at 48hrs (fig1a). This experiment suggested that the best time point to check the effect of siBrm and siBrg1 on cyclinD1 expression was at 48hrs.

Point 5) Fig.4B: The, considering that in both figure 3B and 4C, siBrm cells express myogenin at a level comparable to sictrl cells (Fig. 3B) and are not able to complete the differentiation program. Indeed, a more stringent rescue experiment should rather include another myogenic master gene (i.e. MyoD, as used in the Brm-null MEFs). Is myogenin also up in Brm-null MEF transfected with MyoD? Since MYOD is expressed in BRM knock down cells, as Myogenin, we do not predict that overexpressing it will rescue BRM knock down phenotype.

RE: Despite the reviewer statement that *"inability of myogenin to rescue the differentiation program in Brm knock down cells is to be expected"* this specific issue has never been published or addressed by previous studies and represents one of the key findings of this Ms, which points to the differential gene regulation by Brm and Brg1 and supports the conclusion that Brg1-mediated control of myogenesis relies on the activation of myogenin expression, while Brm-mediated control of myogenesis occurs through a dual regulation of cell cycle genes (repression of Cyclin D1) and muscle genes (activation of late muscle genes, post-myogenin expression).

- **In the Discussion the authors should de-emphasize the relevance of their in vivo findings. Brm null mice have a very weak muscle phenotype. The defective regeneration process upon CTX treatment resulted in a minimal delay in muscle regeneration. These data clearly indicate that there are compensatory mechanisms capable of regulating cell cycle arrest and late skeletal myogenesis in absence of Brm1. The scheme proposed in Fig.6. should be moved to the Supplementary Figures section: there is no evidence, in this manuscript, of the Brg1 and Brm binding ability on myogenin and actn3 regulatory regions.**
  - RE: We agree with the reviewer that in the absence of solid ChIP evidence this scheme is premature. Therefore, it has been removed from the figures.

Minor point: Throughout the manuscript there are numerous typos: i.e. in Fig.1A cycin D1 instead of Cyclin D1, heterogenic instead of heterogenous... The terminology 'genetic ablation' to describe RNA interference experiments is inappropriate.

RE: We truly thank the reviewer for the proofreading and for alerting us on these typos. We have fixed all the points and changed genetic ablation with gene deletion.

2nd Editorial Decision 12 May 2015

Thank you for the submission of your revised manuscript to our journal. We have now received the comments from the referees that were asked to assess it, and both support publication of the study by EMBO reports now. Referee 2 only has a few more suggestions that I would like you to address and incorporate before we can proceed with the official acceptance.

I noticed that the manuscript does not use the correct reference style. Please change it to the numbered EMBO reports style.

Regarding statistics, the number of experiments is not always mentioned and the error bars are not always explained (especially in the supplementary figures), and in the main figure legends it is not clear whether the last sentence regarding statistics refers to all figure panels. Please also specify the statistical test used to calculate p-values in the figure legend (currently missing for Fig 1F, 3B, 4C-E, 6).
in Fig 4D-G you explain the *** in the legend but there are none in the figure panel. In figure 2B n=2, but if n=2 no error bars can be shown. If n is the number of technical repeats please remove the error bars and instead show the single data points along with the mean. Only if the experiment has been independently performed 3 or more times error bars can be calculated.

Please also add scale bars to all microscopy images, there are none in the current version.

Given that you have 6 main figures now, we will publish your paper as an Article and not as a short report. Can you therefore please move all materials and methods to the main manuscript file? We also have now the possibility to publish supplementary figures as "expanded view" files, which means that they will be embedded in the main manuscript file and clickable online (when clicked, they will open in the context of the paper). Therefore, can you please re-label the supplementary figures as expanded view figures EV 1, 2, 3, etc both in the text and in the figure legend and move the figure legends to the end of the main manuscript file? You can also add one more EV figure, as referee 2 suggests. All expanded view figures need to be submitted as separate files. The supplementary table should be called "table EV 1". I am sorry for this re-labeling, but your paper will be one of the first ones with the supplementary figures embedded in the main manuscript, which we think is clearly an improvement in the presentation of supplemental data. There will also be no supplementary information file with your manuscript anymore.

I am looking forward to receiving the final version of your manuscript as soon as possible. Let me know if you have any questions or comments.

REFEREE REPORTS:

Referee #1:

The authors have performed an extensive revision to suitably address the original concerns.

Referee #2:

Response to the points raised by reviewer #2:
I found that all my comments were sufficiently well addressed.
The authors have performed quantitation of Ccnd1 in MEFs, and show the figure "only for reviewers". This is a very informative figure that would fit well within the supplementary section.

I am satisfied with the author's response to my comment regarding the difference of magnitude of the phenotype caused by siBrm (in C2C12) and knock-out of Brm (in mice). I do not see a reason not to insert their response in their discussion, because it is likely a point that many readers will raise.

Response to the points raised by reviewer #3:
The points are well addressed.

The new data presented in Figure 2B are good. However, mathematical conventions dictate that the individual "time points" should not be connected by lines on this graph, because they represent independent conditions rather than a single continuous variable (i.e. in the 3 conditions depicted vary both in terms of time and culture medium composition). A histogram would be the appropriate way of representing these data.

With regards to the comment: "the terminology 'genetic ablation' to describe RNA interference experiments is inappropriate", the authors replied "We have fixed all the points and changed genetic ablation with gene deletion", but RNA interference is not gene deletion either. Typically, researchers use the term "gene knock-down" when RNAi is used.

2nd Revision - authors' response 21 May 2015

We are submitting the revised version of the manuscript entitled “SWI/SNF Brahma is required for cell cycle arrest and late muscle gene expression during skeletal myogenesis.
We appreciated that the reviewer comments were in general positive and supportive, and that reviewers raised a number of constructive comments that were intended to further improve the manuscript.
We have addressed these comments.
We hope that the revised manuscript is now acceptable for publication.

Thank you for your consideration.

Looking forward to receiving your final decision.

3rd Editorial Decision 25 May 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

